Cell Host & Microbe
Resource

Host Metabolism Regulates Intracellular Growth of *Trypanosoma cruzi*

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SUMMARY

Metabolic coupling of intracellular pathogens with host cells is essential for successful colonization of the host. Establishment of intracellular infection by the protozoan *Trypanosoma cruzi* leads to the development of human Chagas' disease, yet the functional contributions of the host cell toward the infection process remain poorly characterized. Here, a genome-scale functional screen identified interconnected metabolic networks centered around host energy production, nucleotide metabolism, pteridine biosynthesis, and fatty acid oxidation as key processes that fuel intracellular *T. cruzi* growth. Additionally, the host kinase Akt, which plays essential roles in various cellular processes, was critical for parasite replication. Targeted perturbations in these host metabolic pathways or Akt-dependent signaling pathways modulated the parasite's replicative capacity, highlighting the adaptability of this intracellular pathogen to changing conditions in the host. These findings identify key cellular process regulating intracellular *T. cruzi* growth and illuminate the potential to leverage host pathways to limit *T. cruzi* infection.

INTRODUCTION

Chagas' disease is a progressive and debilitating parasitic disease that develops over decades in a subset of individuals chronically infected with the obligate intracellular protozoan *Trypanosoma cruzi*. Current global estimates indicate that 8–11 million people are infected with *T. cruzi*, primarily in rural Latin America, and of these, roughly 3 million will develop life-threatening, incurable chronic Chagasic syndromes (World Health Organization, 2012). Parasite persistence in cardiac and smooth muscle, with accompanying inflammation and immune dysregulation, plays an essential role in the pathogenesis of Chagas' disease (Gutierrez et al., 2009; Tarleton, 2001), where links to host metabolic perturbations are emerging (Garg et al., 2004; Machado et al., 2011). *T. cruzi* infection impacts cardiac energy metabolism (Garg et al., 2004) in which attenuated mitochondrial function in the heart is associated with the development and progression of Chagasic cardiomyopathy (Wen et al., 2004). Moreover, persistent *T. cruzi* infection of adipose tissue in chronic Chagas' patients (Ferreira et al., 2011) predicts long-term consequences of the associated inflammation on host glucose and lipid metabolism, which may influence disease progression. As the currently available drugs to treat Chagas' disease exhibit toxicity and fail to clear intracellular parasites in the chronic stage of infection, the need to develop treatments for this neglected disease is paramount.

The vertebrate stages of *T. cruzi* consist of nondividing "trypomastigote" forms that actively penetrate a wide variety of host cell types to establish intracellular infection and "amastigote" forms that proliferate in the host cell cytoplasm. The current model of the *T. cruzi* trypomastigote invasion process was derived from the collective contribution of several laboratories over the past two decades (reviewed in Caradonna and Burleigh, 2011). In contrast to these efforts, the cellular and metabolic processes required to fuel the growth of intracellular *T. cruzi* amastigotes remain largely unknown (Gutteridge and Gaborak, 1979; Inbar et al., 2012; Robello et al., 1997; Ullman and Carter, 1997). Thus, a global determination of the components, function, and consequence of host cellular pathways that are exploited by intracellular *T. cruzi* growth and survival will have significant implications for the understanding and treatment of chronic Chagas' disease.

Toward this end, we have performed a genome-wide RNA interference (RNAi) screen targeting genes in mammalian host cells to identify cellular processes that support intracellular *T. cruzi* infection. Host factors associated with *T. cruzi* infection identified in a recent study (Genovesio et al., 2011) did not identify the primary points of action. A critical element of our screening strategy is the inclusion of secondary screens designed to discriminate host functional pathways that impact trypomastigote invasion and the prereplication phase (<24 hr postinfection) from host processes that support intracellular *T. cruzi* amastigote growth (>24–90 hr). Consistent with the current view of the *T. cruzi* invasion process, we find that establishment of intracellular residence by this pathogen is largely influenced by host cell signaling molecules and cytoskeletal proteins. In contrast, the intracellular replicative phase of the *T. cruzi* infection cycle is supported by host metabolic networks and cellular signaling pathways, a subset of which were validated with small molecule inhibitors and biochemical rescue experiments. Thus, the ability to modulate the intracellular

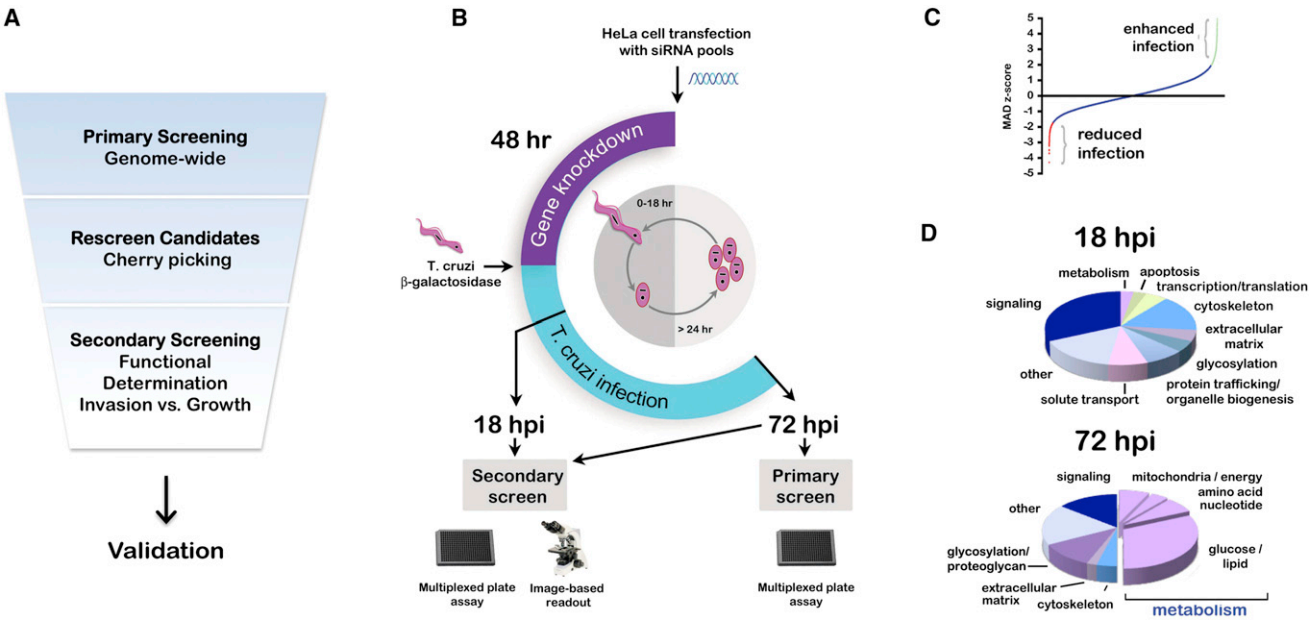

Figure 1. Genome-wide siRNA Screen Differentiates Host Processes Important for *Trypanosoma cruzi* Invasion and Growth

(A) Overall strategy for the three-phase RNAi screen.

(B) Schematic for set up of *T. cruzi* growth assay and primary and secondary screen readouts. Note that secondary screens exploited both the multiplexed plate assay and an image-based readout in parallel with endpoints of 18 hpi and 72 hpi, to discriminate effects of host gene knockdown on intracellular parasite growth from prereplication events in the parasite life cycle (illustrated in circular inset).

(C) Ranked MAD z scores of relative infection data from primary screen.

(D) Functional breakdown of secondary screen hits that impact intracellular parasite infection (18 hr) and growth (72 hr) from Table S2, manually curated from UniProt.

See also Figure S1, Table S1, and Table S2.

replicative capacity *T. cruzi* amastigotes by targeting host cellular and metabolic pathways offers an experimental tool that can be exploited to probe the intimate connections forged between *T. cruzi* and its mammalian host cell.

RESULTS

A Genome-wide RNAi Screen in Mammalian Host Cells Identifies Interconnected Metabolic Pathways as Key Regulators of Intracellular *T. cruzi* Growth

To gain functional insights into host cellular pathways exploited by *T. cruzi* during the establishment and maintenance of intracellular infection, we established a three-phase RNAi screen in mammalian cells (Figure 1A). In the primary screening phase, an arrayed human small interfering RNA (siRNA) library consisting of 25,586 siRNA pools (four individual siRNAs/pool) was assayed in HeLa cells to determine the impact of host cell gene silencing on *T. cruzi* infection with a multiplexed end-point parasite growth assay (Figure 1B and Figure S1 available online). This assay permitted the simultaneous measurement of β-galactosidase-expressing *T. cruzi* (Beta-Glo) and host cell abundance (Cell Titer-Fluor) as detailed in the Experimental Procedures. For each well, the median absolute deviation (MAD) for the BetaGlo/Cell Titer-Fluor ratios was derived and used as the first criterion for assigning hits: a MAD z score of ≤ -1.75 or ≥ 2.5. Wells in which host cell abundance was $\leq 40\%$ of nontargeting siRNA controls were excluded. Based on these criteria, the primary screen yielded 359 siRNA pools (1.69% of total) that restrict *T. cruzi* infection and 293 siRNA pools (1.38%) that enhance intracellular parasite burden (Figure 1C and Table S1). Focusing primarily on siRNA pools that caused an overall reduction in the intracellular burden of *T. cruzi*, we rescreened 240 selected pools in the parasite growth assay (Figure 1B) as four individual siRNAs deconvoluted from the original pool. We find that 81% of the siRNA pools contained at least one siRNA that reproduced the original effect on parasite infection (Table S1). At the rescreening stage, a few additional siRNAs that did not make the significance cutoffs in the primary screen, but which represented "borderline" hits or genes involved in emerging pathways of interest, were included. Genes with unknown function were not considered for further testing. Efficient gene silencing in HeLa cells was observed for the siRNA pools tested (Figure S2A).

To discriminate host factors that influence early steps in the establishment of intracellular *T. cruzi* infection (invasion, vacuole biogenesis, and cytosolic localization) from factors regulating intracellular growth of cytosolic *T. cruzi* amastigotes, we conducted secondary screens to measure relative parasite infection levels at pre- and postreplication time points (18 and 72 hours postinfection [hpi], respectively) (Figures 1B and S2B). In accordance with their distinct roles in the parasite life cycle, host genes associated with *T. cruzi* invasion and intracellular replication (Table S2) displayed discrete patterns of functional enrichment (Figure 1D). Our results reveal that the prereplication phase of the *T. cruzi* infection cycle is predominantly affected by host

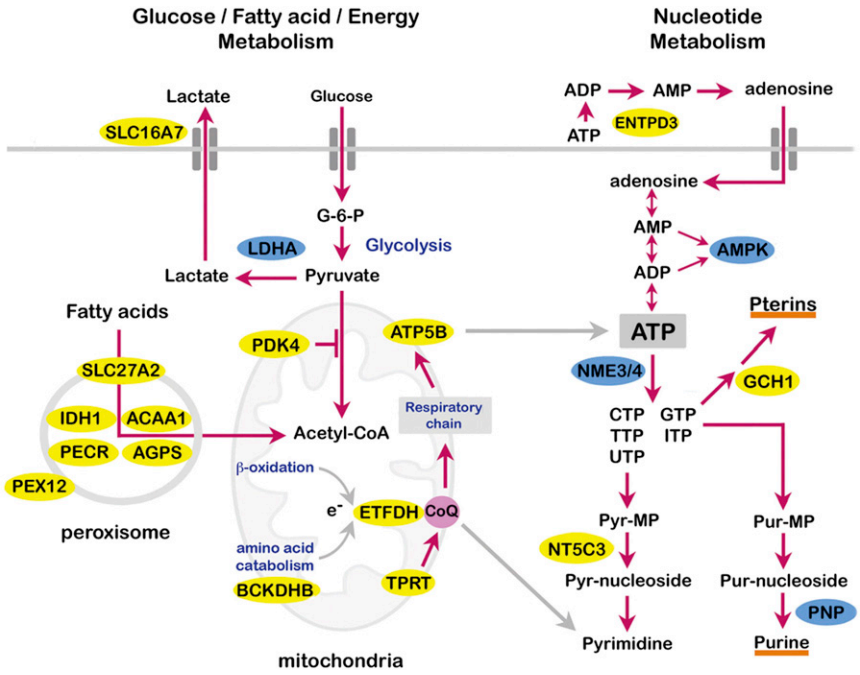

Figure 2. Schematic of Interconnected Host Metabolic Pathways that Influence Intracellular Growth of *T. cruzi* Amastigotes

Highlighted are host genes that, when silenced with specific siRNA pools, reduce (yellow) or enhance (blue) intracellular growth of *T. cruzi* amastigotes. Underlining indicates pathways for known *T. cruzi* auxotrophies. See also Figure S2.

cell signaling pathways and cytoskeletal regulators (Table S2), in line with our current understanding of the parasite invasion process (Caradonna and Burleigh, 2011). In contrast, host factors associated with *T. cruzi* amastigote growth in mammalian cells were highly enriched for metabolic functions (Figure 1D and Table S2). Despite functional leads to the parasite internalization process, we concentrated on host pathways that impact the intracellular growth of *T. cruzi* amastigotes, for which little is currently known. Within this group, we focused on the subset with the highest enrichment and most promising pharmacological targets, specifically Akt signaling and hits related to glucose, lipid, nucleotide, and energy metabolism (Figure 2).

Perturbations in Host Glucose and Fatty Acid Metabolism Alter Intracellular Parasite Growth Rates

In mammalian hosts, *T. cruzi* replicates and persists within tissues that exhibit high rates of fatty acid metabolism (Fritz, 1961), such as cardiac muscle, smooth muscle, and adipose (Brener, 1973; Combs et al., 2005; Macedo and Pena, 1998; Ferreira et al., 2011). The rate of fatty acid oxidation in the mitochondria is controlled by the flux of glucose through the glycolytic pathway and the rate of conversion of pyruvate to acetyl CoA by pyruvate dehydrogenase (PDH). *T. cruzi* infection of cardiac tissue triggers early upregulation of host fatty acid β oxidation genes followed by downregulation of the PDH complex (Garg et al., 2004). As a negative regulator of PDH, pyruvate dehyrogenase kinase 4 (PDK4) functions as the main gatekeeper of the balance between glucose and fatty acid oxidation (Sugden et al., 2001). Here, we find that *T. cruzi* growth is poorly supported in *PDK4*-depleted cells (Figure 3A) that display increased PDH activity (Figure 3B) and decreased fatty acid oxidation (Figure 3C). Consistent with the idea that increased flux of pyruvate through PDH contributes to a cellular environment that is restrictive for *T. cruzi* amastigote growth, we find that intracellular replication of *T. cruzi* is enhanced in primary human fibroblasts that exhibit reduced PDH activity (80% reduction) (data not shown; Constantopoulos et al., 1986) (Figure 3D). Predicted consequences of decreased PDK4 levels and increased PDH activity include a decrease in cellular pyruvate pools and a shift in the fuel utilization balance from fatty acid oxidation toward glucose oxidation (Figure 3E) (Sugden et al., 2001). As pyruvate supplementation of the growth medium fails to rescue *T. cruzi* growth in *PDK4*-depleted cells (data not shown), the interaction of intracellular *T. cruzi* amastigotes with host pyruvate metabolism may be more complex than the fulfillment of a nutritional requirement. (Note that pyruvate uptake by *T. cruzi* has not been demonstrated.) Instead, *T. cruzi* amastigotes may display a growth advantage under conditions where host cell fatty acid oxidation is favored over glucose utilization.

In mammals, β oxidation occurs in the peroxisomes and the mitochondria (Poulos, 1995). Very-long-chain fatty acids (VLCFAs; $\geq C_{22}$) are transported into peroxisomes and oxidized to produce shorter-chain fatty acids that are shuttled to the mitochondria for continued oxidation by a number of acyl-CoA dehydrogenases (ACADs) to ultimately produce acetyl CoA (Ghisla and Thorpe, 2004). Consistent with the notion that *T. cruzi* amastigotes flourish in an environment that favors fatty acid oxidation, peroxisomal proteins involved in activation/transport of VLCFAs (SLC27A2) and β oxidation (ACAA1 and IDH1) were identified in our screen as positively associated with intracellular *T. cruzi* growth (Figure 2). In addition, primary human fibroblasts deficient in mitochondrial ACADVL (Fang et al., 2000), which accommodates substrate acyl chains of ($\geq C_{16}$), sustain significantly lower levels of intracellular *T. cruzi* growth (Figure 3D). In contrast, parasite growth restriction was not observed in cells deficient for medium- or short-chain ACADs (data not shown). Combined, these data identify long-chain fatty acid oxidation as a key process in mammalian host cells associated with intracellular *T. cruzi* growth. The upregulation of fatty acid utilization pathways in axenically derived *T. cruzi* amastigotes (Atwood et al., 2005) indicates the likelihood that fatty acid metabolism in the intracellular parasite is coupled with that of their mammalian hosts. Whether *T. cruzi* amastigotes avail of fatty acid intermediates generated in host peroxisomal or mitochondrial oxidative pathways or whether they benefit indirectly from the production of energy and reductive intermediates formed in these catabolic processes remains to be determined.

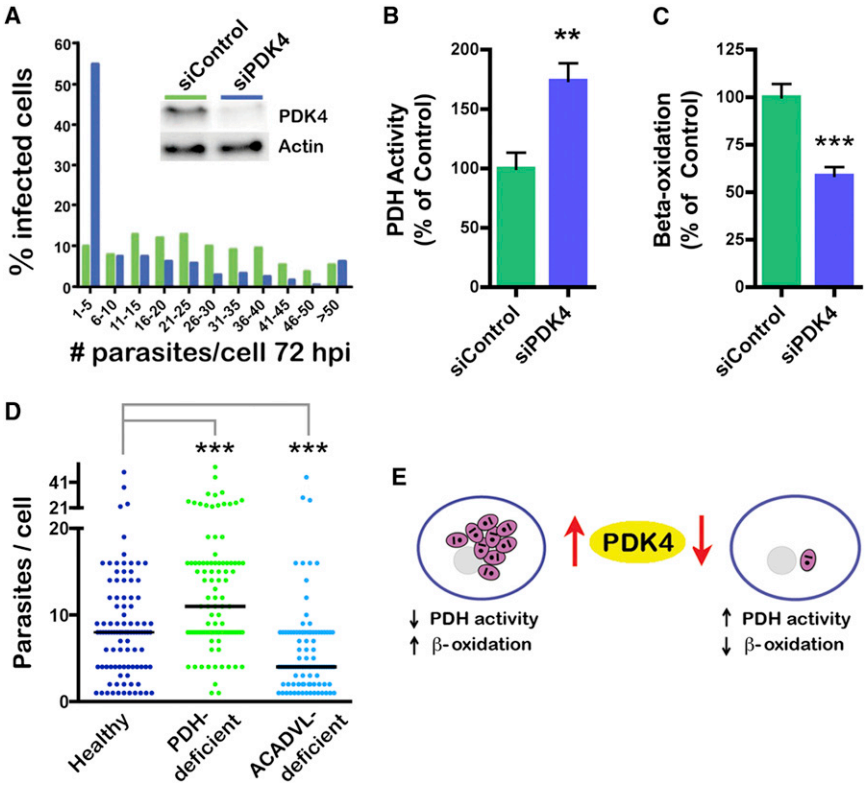

Figure 3. Intracellular *T. cruzi* Growth Is Coupled to Host Fatty Acid Metabolism

(A) Western blot of knockdown (inset) and distribution data for the number of intracellular parasites in HeLa cells transfected with nontargeting siRNA (siControl) or siPDK4 at 72 hpi. Bar graph values were derived from image-based analysis of secondary screening data (Table S2).

(B and C) PDH activity (B) and β oxidation (C) measured in HeLa cells transfected with nontargeting (siControl) or siPDK4 (n = 6). Error bars indicate the standard error (**p < 0.01, ***p < 0.001).

(D) Primary human fibroblasts deficient in pyruvate dehydrogenase (PDH) or acyl-CoA dehydrogenase (very-long chain) ACADVL were infected with *T. cruzi* and counted 48 hpi. Scatter plots of numbers of parasites/infected cell for 100 infected cells (per cell line) with the median highlighted (black line) are shown. Significance was determined by a Kruskal-Wallis test followed by Dunn's Multiple Comparison test to directly compare infection of metabolically deficient cells to healthy control (***p < 0.001). Experiment was repeated three times with similar results.

(E) Schematic of balance between PDH regulation and fatty acid oxidation and its effects on parasite growth.

Parasite Growth Is Dependent on the Availability of Host-Derived Cofactors and Metabolic Products

In mammalian cells, electrons generated via fatty acid β oxidation and amino acid catabolism in the mitochondria are transferred to Coenzyme Q_{10} (CoQ_{10}) via the electron transfer flavoprotein dehydrogenase ETFDH (Figure 2). Limitation of CoQ_{10} production in host cells by silencing of the biosynthetic enzyme transprenyltransferase (*TPRT*) restricts intracellular amastigote growth (Figure 4A). The parasite growth defect is completely restored with CoQ_{10} supplementation of the culture medium (Figure 4B). In addition to its role in mitochondrial energy production, CoQ_{10} is critical for de novo pyrimidine synthesis (López-Martín et al., 2007) and exhibits antioxidant properties (Jeya et al., 2010). *T. cruzi* growth in *TPRT* knockdown cells is partially restored with the addition of uridine but not after supplementation with the antioxidant N-acetylcysteine (NAC) (Figure 4B). These data indicate that CoQ_{10} deficiency in the host restricts *T. cruzi* growth by limiting the availability of pyrimidines. While *T. cruzi* has pyrimidine synthetic capability (Gutteridge and Gaborak, 1979), our host functional data imply that intracellular amastigotes are reliant on host pyrimidine pools to support their growth. Consistent with this observation, limitation of pyrimidine nucleoside and/or nucleobase generation downstream of the host cytosolic nucleotidase, NT5C3, is suboptimal for intracellular *T. cruzi* growth (Figure 2).

Purines and pteridine auxotrophy (Gutteridge and Gaborak, 1979; Robello et al., 1997) predicts that intracellular *T. cruzi* amastigotes will scavenge these essential nutrients from the host cell cytosol. As biochemical data for the amastigote forms of *T. cruzi* are limited (Gutteridge and Gaborak, 1979; Nakajima-Shimada et al., 1996), the functional links to host purine and pteridine metabolic pathways uncovered in our unbiased RNAi screen were particularly valuable (Figure 2). Limitation of tetrahydrobiopterin production in host cells by silencing of a key biosynthetic enzyme GTP cyclohydrolase 1 (*GCH1*) restricts intracellular *T. cruzi* growth (Figure 4C) where the parasite growth defect is rescued after biochemical supplementation of *GCH1*-depleted cells with 7,8 dihydro-L-biopterin (Figure 4D). These findings provide functional evidence that intracellular *T. cruzi* amastigotes are tightly coupled to the biopterin biosynthetic capabilities of the host cell. Purines are acquired by trypanosomatids from the environment via members of parasite-encoded equilibrative nucleoside transporters (Landfear, 2008) (http://tritrypdb.org/tritrypdb/). Silencing of purine nucleoside phosphorylase (*PNP*) in host cells has the predicted consequence of limiting purine nucleoside to nucleobase conversion. As such, the associated increase in *T. cruzi* amastigote growth in *PNP*-depleted cells was striking (Figure 2) and leads to the testable hypothesis that intracellular stages of *T. cruzi* preferentially transport purine nucleosides. Combined with functional evidence that host pyrimidines are critical for fueling intracellular amastigote growth (Figure 4B), despite the capacity for de novo synthesis (Gutteridge and Gaborak, 1979), data from our RNAi screen provide functional evidence for the reliance of *T. cruzi* amastigotes on host purine and pyrimidine pools as suggested by inhibitor studies (Nakajima-Shimada et al., 1996). These findings may have important implications considering purine and pyrimidine salvage pathways in intracellular *T. cruzi* amastigotes as potential therapeutic targets.

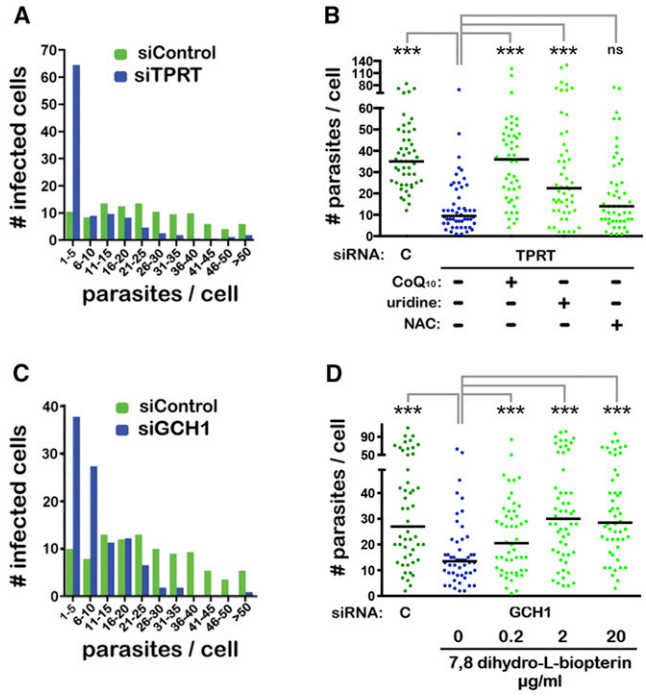

Figure 4. Modulation of Perturbed Pathways with Metabolite Supplementation Restores Parasite Growth

(A) Distribution data for the number of intracellular *T. cruzi* in HeLa cells transfected with nontargeting siRNA (siControl) or siRNAs targeting transprenyltransferase *TPRT* (siTPRT), which is required for CoQ_{10} synthesis.

(B) Scatter plots of intracellular parasites in infected cells transfected with nontargeting control (siControl) or siTPRT supplemented with CoQ_{10} (5 μM), uridine (10 μM), or N-acetylcysteine (NAC; 1 mM).

(C) Distribution data for the number of intracellular parasites in HeLa cells transfected with nontargeting siRNA (siControl) or siRNAs targeting GTP cyclohydrolase 1 (*GCH1*), the first enzyme in the biopterin synthesis pathway.

(D) Supplementation of growth medium with 7,8 dihydro-L-biopterin rescues *T. cruzi* growth defect in *GCH1*-depleted cells.

(B and D) Black lines indicate the count median for each treatment. Significance was determined between control unsupplemented *TPRT*- or *GCH1*-deficient cells (blue) and other conditions (green) by a Kruskal-Wallis test followed by Dunn's Multiple comparison test (***$p < 0.001$). Scatter plot distributions shown are representative of three independent experiments.

Homeostatic mechanisms that maintain high ATP/ADP ratios are critical for cell viability and growth. As such, preservation of cellular nucleotide pools is likely to present a fundamental challenge for *T. cruzi*-infected host cells. The majority of cellular ATP is generated in the mitochondria in a reaction that is coupled to oxidative phosphorylation and the transfer of electrons generated via glucose, fatty acid, and amino acid catabolism. Host genes participating in these processes emerged in our secondary screen as strongly associated with intracellular *T. cruzi* amastigote growth (Figures 1D and 2 and Table S2), including a catalytic subunit of the mitochondrial ATP synthase, *ATP5B*. Limitation of the conversion of cytosolic ATP to other nucleoside triphosphates via transphosphorylation reactions catalyzed by nucleoside diphosphate kinases, such as NME3 and NME4, significantly boosts intracellular *T. cruzi* growth (Figure 2 and Table S1), suggesting that maintenance of cellular ATP/ADP ratios provides a distinct advantage for the parasite that could include keeping the activity of AMPK in check (as demonstrated in Figure 5H, acute silencing of AMPK catalytic or regulatory subunits provides a more favorable growth environment for intracellular *T. cruzi*). Viewed within the larger context of nucleotide metabolism, these data highlight critical nodes in host bioenergetic and nutrient-generating pathways to which cytosolically localized *T. cruzi* amastigotes couple to meet metabolic needs.

Genetic and Pharmacological Targeting of Host Akt Signaling Inhibits Intracellular *T. cruzi* Replication

The serine/threonine kinase, Akt, participates in an extensive cellular network that controls multiple pathways, including prosurvival responses, glucose metabolism, and nutrient sensing via the mTORC1 pathway (Huang and Manning, 2009; Weichhart, 2012). Akt is activated in a variety of mammalian host cells during the *T. cruzi* invasion process (Chuenkova et al., 2001; Woolsey et al., 2003) prompting a prosurvival response in infected host cells that is sustained throughout the intracellular parasite growth cycle (Chuenkova and PereiraPerrin, 2009). Here, our genome-wide RNAi screen has uncovered a role for host Akt as a regulator of intracellular *T. cruzi* growth. Acute silencing of *AKT1* restricts intracellular *T. cruzi* growth in HeLa cells without discernable effects on prereplication events in the parasite life cycle (Figures 5A–5C). Whereas isoform specificity for *AKT1* was observed in HeLa cells, silencing of both *AKT1* and *AKT2* in mouse embryonic fibroblasts (MEFs) and human dermal fibroblasts (HFFs) was necessary to observe comparable effects on *T. cruzi* growth (Figures S3A and S3B). The availability of highly selective inhibitors of AKT1/2 isoforms (Green et al., 2008) permits chemical validation of Akt signaling as a key host cell pathway regulating intracellular *T. cruzi* replication. Pharmacological targeting of host AKT1/2 with the allosteric inhibitor AktVIIIi restricts intracellular parasite growth and signaling (Figures 5D and 5E) in a range of mammalian cell types (Figure S3C), with the exception of *AKT1/2*-deficient MEF in which residual *T. cruzi* growth is refractory to AktVIIIi (Figure 5F). To determine whether targeting of host Akt inhibits the replicative capacity of the parasite, we established a proliferation assay for intracellular *T. cruzi* amastigotes based on flow cytometric analysis of CFSE stained parasites (Figure 5G). In untreated cells, five generations of intracellular *T. cruzi* amastigotes can be resolved by 48 hr postinfection. Treatment of infected fibroblasts with AktVIIIi at 18 hpi (immediately prior to the first division of intracellular parasites) resulted in significant inhibition of parasite proliferation following an initial round of parasite cell division (Figure 5G). Thus, pharmacological targeting of host Akt demonstrates the proof-of-principle concept that unbiased functional screens are valuable tools for the identification of cellular targets in mammalian host cells that, when inhibited pharmacologically, restrict intracellular *T. cruzi* infection. This principle can be applied to any druggable molecular target that emerges from this or similar screening approaches.

T. cruzi Growth Is Enhanced by Manipulation of Anabolic Growth Pathways

The mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is an Akt-dependent pathway that senses cellular

nutrient, energy, and redox status and controls protein synthesis (Huang and Manning, 2009; Weichhart, 2012). mTORC1 signaling is inhibited by nutrient and growth factor deficiency or by treatment with the small-molecule inhibitor rapamycin. Perturbations in the host mTORC1 pathway in *T. cruzi*-infected cells revealed in transcriptomic studies (Costales et al., 2009) suggest that *T. cruzi* intersects this host pathway, directly or indirectly. Consistent with this suggestion, acute silencing of genes encoding catalytic (*PRKAA1*) and noncatalytic (*PRKAB1*) subunits of AMP kinase (AMPK), a negative regulator of mTORC1, significantly enhance intracellular growth of *T. cruzi* (Figure 5H, Table S1, and Table S2). To better assess the impact of mTORC1 on parasite replication, we exploited a HeLa cell line stably expressing short hairpin RNA (shRNA) targeting tuberous sclerosis protein 2 (*TSC2*), an Akt substrate and a negative regulator of mTORC1 for infection studies. We find that *T. cruzi* growth is insensitive to rapamycin in control cells; however, constitutive activation of mTORC1 in shTSC2 cells (Huang et al., 2008) fueled increased intracellular *T. cruzi* growth in a rapamycin-sensitive manner (Figure 5I). The observation that both basal and mTORC1-stimulated *T. cruzi* growth are sensitive to AktVIIIi, whereas only the accelerated parasite growth in *TSC2*-depleted cells is sensitive to rapamycin (Figure 5I), exposes the plasticity of parasite populations with respect to their ability to access mTORC1-dependent and independent pathways downstream of Akt to support replication in mammalian cells (Figure 5J). The ability of *T. cruzi* to integrate its metabolic needs with different host cellular pathways would offer a level of flexibility to facilitate parasite survival in the face of changing environmental conditions encountered as the parasite adapts to life in multiple hosts and cell types in nature.

DISCUSSION

At the outset of the present study, we had little prior knowledge of host cellular pathways that function to support intracellular infection by the obligate intracellular parasite, *Trypanosoma cruzi*. As a result of the unbiased functional screen and accompanying experimental validation reported here, we are now able to place the predicted metabolic dependencies of *T. cruzi* (Gutteridge and Gaborak, 1979; Inbar et al., 2012; Ullman and Carter, 1997) within a larger interconnected framework encompassing nucleotide metabolism, glucose/fatty acid metabolism, host energetics, and Akt signaling. The identification of biochemical pathways in our screen that yield products known to be required for *T. cruzi* growth and replication (i.e., purine and biopterin) provides a high level of confidence in the functional associations that have emerged in the screen.

A functional link between host CoQ_{10} production, pyrimidine biosynthesis, and *T. cruzi* amastigote growth was exposed in our unbiased siRNA screen. Acute silencing of host *TPRT*, which is required for CoQ_{10} biosynthesis, dramatically inhibited the growth of intracellular amastigotes without exerting a detrimental effect on the host cell. In biochemical supplementation experiments, we were able to show that addition of CoQ_{10} was sufficient to rescue parasite growth. The ability to restore parasite growth with uridine supplementation as well suggests that the main requirement for CoQ_{10} is associated with its activity as a cofactor for the pyrimidine biosynthetic enzyme, dihydroorotate dehydrogenase. Considering that *T. cruzi* has the capacity to synthesize pyrimidines de novo, the inferred reliance on host pyrimidine pools was not expected. Collectively, these findings highlight the utility of a functional genomics approach to identify critical regulators of the dynamic *T. cruzi*-host interaction.

The emergence of the host serine/threonine kinase Akt as a regulator of *T. cruzi* amastigote replication was unexpected, considering the extensive focus on Akt as a key mediator of the prosurvival response triggered by *T. cruzi* (Chuenkova and PereiraPerrin, 2009). The coupling of *T. cruzi* amastigote replication to a critical regulator of host cell survival suggests a sophisticated strategy for establishment of long-term infection in the host. It should be noted that *T. cruzi* is not unique in its exploitation of host Akt-signaling pathways (Kuijl et al., 2007; Kumar et al., 2010), where its influence on host phagolysosomal fusion events is manipulated by intracellular bacteria (Kuijl et al., 2007). The mechanistic basis for the influence of host Akt on *T. cruzi* replication remains to be determined. However, given its wide functional reach in mammalian cells, including a prominent role in glucose and lipid metabolism (Hay, 2011), the influence of host Akt on intracellular *T. cruzi* replication is likely to be multifactorial.

Several observations suggest that intracellular *T. cruzi* amastigotes benefit from a host cell metabolic environment that favors fatty acid oxidation over glucose oxidation. Acute silencing of *PDK4*, the main gatekeeper of the balance between glucose and fatty acid utilization, inhibits amastigote growth, whereas parasites exhibit a growth advantage in cells with PDH deficiency. Host genes involved in very-long-chain fatty acid oxidation in host peroxisomes were identified in the screen as influencing parasite growth, and cells with defective mitochondrial fatty acid oxidation were less competent to support intracellular *T. cruzi* growth. Proteomic evidence shows that the amastigote form of the *T. cruzi* life cycle upregulates the capacity for fatty acid uptake and oxidization (Atwood et al., 2005), suggesting the natural coupling of *T. cruzi* growth to fatty acid metabolism in the host.

Alongside functional insights emerging from our study, our observations suggest a level of plasticity in *T. cruzi* amastigotes populations in which intracellular parasite growth rates are responsive to environmental cues. While this is a highly intuitive concept—i.e., restricting "nutrient" pools should slow intracellular parasite growth—our study provides experimental evidence that intracellular *T. cruzi* growth can be significantly altered (↑ or ↓) simply by perturbing host cellular functions. This flexibility is further exemplified by the differential "usage" of host Akt-dependent pathways, both mTORC1-dependent and -independent, to fuel intracellular *T. cruzi* amastigote growth, exposing layers of redundancy with respect to host pathway utilization by the parasite. The capacity for adaptation would be especially relevant in the context of a dynamic natural infection in the mammalian host. Plasticity within parasite populations, reflected in an ability to alter growth rates to match particular metabolic microenvironments, may be a critical mechanism underlying tissue tropism and persistent infection. Selective persistence of *T. cruzi* in muscle and adipose tissue presents the possibility that cellular metabolism characteristic to these tissues, such as an increased reliance on fatty acid metabolism for energy production (Fritz, 1961), offers a selective advantage

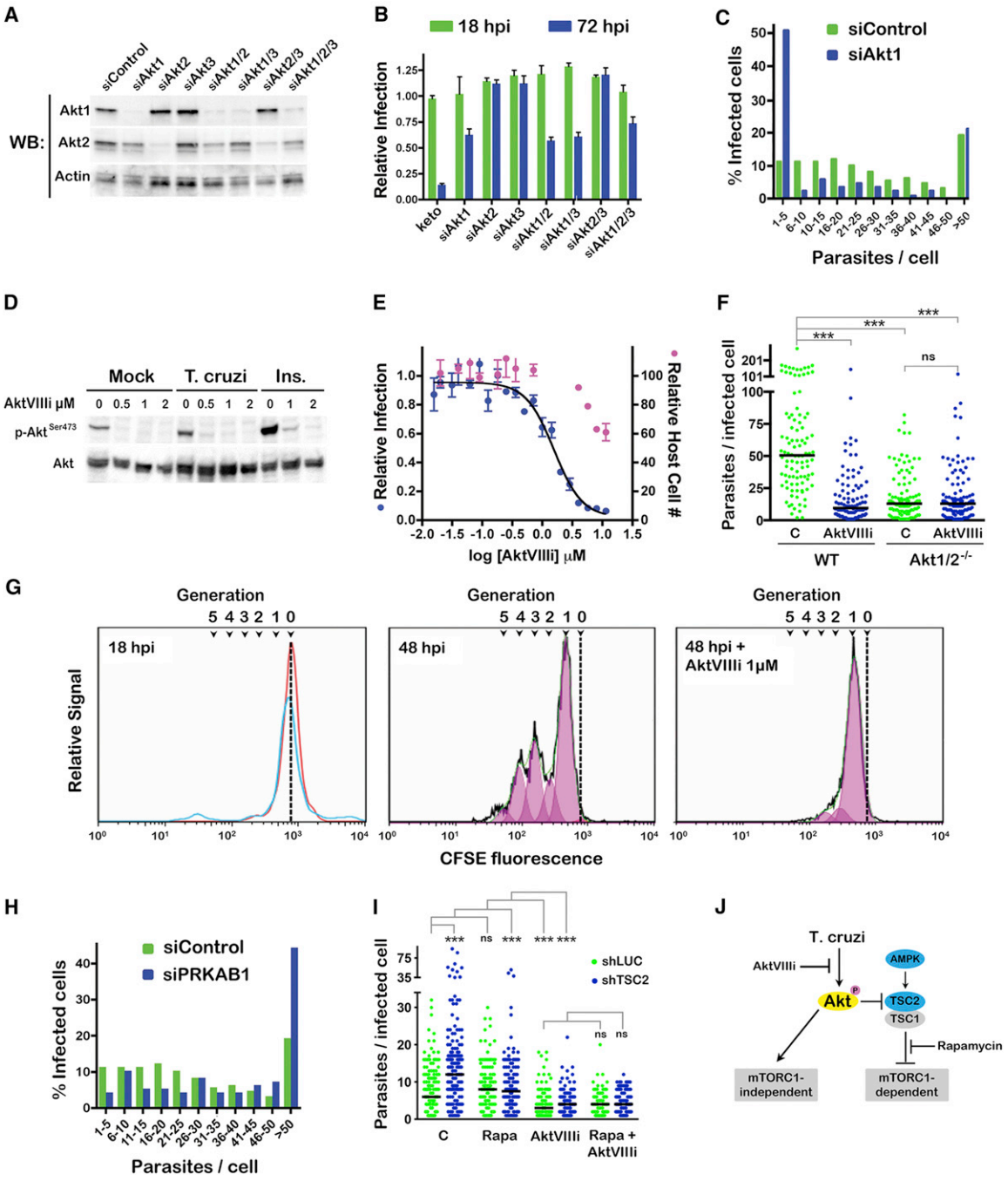

Figure 5. Host Cell Akt Regulates Intracellular *T. cruzi* Growth via mTORC1-Dependent and -Independent Mechanisms

(A) Western blot of AKT1 and AKT2 expression levels in HeLa cells as compared to β-actin, after siRNA-mediated silencing of *AKT1*, *AKT2*, and *AKT3* isoforms singly and in combination.

(B) Silencing of *AKT1* in HeLa cells inhibits intracellular *T. cruzi* growth (72 hpi) but not the ability to establish intracellular infection (18 hpi) as determined with the multiplexed plate assay (detailed in the Experimental Procedures) and compared to nontargeting control siRNA. Data are represented as the mean ± SD ($n \geq 3$).

(C) Distribution plots of intracellular *T. cruzi* amastigotes (number of parasites/infected cell) in HeLa transfected with nontargeting control siRNA (siControl) or after acute silencing of *AKT1* (siAkt).

(D) Relative phospho-Akt^{Ser473} levels in HeLa cells determined by western blot in mock-infected, *T. cruzi*-infected, or insulin-stimulated (Ins) cells 72 hpi in 0.5% serum-containing media. AktVIIIi was added to cells at 18 hpi.

(E) Dose-dependent inhibition of *T. cruzi* growth in HeLa cells treated with AktVIIIi at 18 hpi and relative infection measured at 72 hpi with the multiplexed plate assay. Values represent the mean ± SD ($n \geq 3$).

(F) *T. cruzi* growth in *AKT1/2* knockout MEFs measured at 72 hpi is significantly lower than in WT controls and refractory to 1 μM AktVIIIi added at 18 hpi.

(legend continued on next page)

to the parasite enabling it to survive in the face of an otherwise punishing immune response.

Despite suggestions of flexibility and adaptation to changing environmental conditions, *T. cruzi* growth in mammalian cells is highly susceptible to targeted perturbations in host metabolic and signaling functions. As such, pharmacological targeting of host metabolism to uncouple intracellular *T. cruzi* from its nutritional dependencies may prove an effective strategy for controlling pathogen spread. The concept of host-targeted therapies is not new for virologists (Ikeda and Kato, 2007; Schols, 2004) and is gaining traction for nonviral pathogens (Jayaswal et al., 2010; Kuijl et al., 2007). Drug discovery efforts for Chagas' disease have focused primarily on the identification of target pathways in the parasite (Buckner and Navabi, 2010). However, with inherent heterogeneity in drug sensitivity among *T. cruzi* isolates/strains (e.g., Bustamante and Tarleton, 2011), the consideration of host-targeted therapies for Chagas' disease as an alternative to, or in combination with, antitrypanosomal compounds is clearly warranted. This study opens an opportunity in the Chagas' disease field to exploit host genetics and small molecules to gain fundamental insights into the *T. cruzi* infection process with a view toward the identification of pharmacological targets in the parasite and the host.

EXPERIMENTAL PROCEDURES

siRNA Screen

HeLa cells were reverse transfected with arrayed siRNA SMARTpools (50 nM) (Thermo Fisher Scientific, Lafayette, CO) complexed in Oligofectamine reagent (Invitrogen). At 48 hr posttransfection, cells were infected with *T. cruzi* multiplicity of infection (MOI) 5 for 2 hr, followed by two washes with PBS and incubation in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 2 mM L-glutamine (D2) (phenol free) for a total of 72 hr. Each assay plate also included ketoconazole (15 μM) as a positive control for inhibition of *T. cruzi* growth, siRNA to polo-like kinase1 (*PLK1*) as a control for transfection and cell death, and nontargeting siControl (Dharmacon siRNA#2). The screen was performed in triplicate or duplicate according to plate content, i.e., library plates enriched in pseudogenes or genes of unknown function were screened in duplicate.

Multiplexed *T. cruzi* Infection Assay

Trypanosoma cruzi Tulahúen strain stably expressing β-galactosidase (generously provided by Fred Buckner, University of Washington) was maintained by weekly passage in $LLcMK_2$ cells (ATCC) as described (Woolsey et al., 2003). Relative intracellular *T. cruzi*-β-galactosidase infection and host cell viability were measured in a multiplexed plate assay with CellTiterFluor (Cell Viability Assay; Promega, Madison, WI) and Beta-Glo reagent (Promega), per the manufacturers' protocol, measured on the EnVision Plate Reader (Perkin Elmer). The MAD for the ratio of BetaGlo/Cell Titer-Fluor (LUM/INTENS) values measured in each well for each screening plate was calculated. The MAD z score [z = (x − m) / (MAD * K)] was calculated for each SMARTpool on the plate, where x is the LUM/INTENS for each well, m is the median LUM/INTENS of the plate, MAD is the median absolute deviation of the LUM/INTENS ratios on the plate, and K is the scale factor 1.4826. A SMARTpool was further considered if the MAD z score was ≤ -1.75 or ≥ 2.5 and $\geq 40\%$ host cell viability (%v = [(x – background) / (plate ave – background)]) of each well, where x is the intensity value for the well, "background" is the average PLK1 intensity for the plate, and "plate ave" is the average intensity for the plate. In assays where the plate components were not pinned at random, as in the cherry picked and secondary assays, the LUM/INTENS was compared to the LUM/INTENS of control siRNA2. A SMARTpool was considered a hit if the relative ratio $[(\text{LUM/INTENS})^{\text{target siRNA}} / (\text{LUM/INTENS})^{\text{control siRNA}}]$ scored ≤ 0.66 and fulfilled the $\geq 40\%$ cell viability cutoff established above.

Secondary Screens

For secondary screens, custom plates containing selected siRNA pools (purchased from Dharmacon; Thermo Fisher Scientific, Lafayette, CO) targeting annotated genes in pathways that emerged as hits in the primary or rescreening phases of the genome-wide screen. HeLa cells were transfected as above, with the exception that 25 nM (final) siRNA was assayed per well and each siRNA pool was assayed in three independent *T. cruzi* infection experiment with at least two biological replicates per experiment. Mean LUM/INTENS values were determined across experiments for each siRNA SMARTpool that was considered a candidate "hit" if the relative ratio scored ≤ 0.66 (comparing target to control siRNA, as above) and fulfilled the $\geq 40\%$ host cell viability cutoff at 18 or 72 hpi. In addition to the multiplexed plate assay, a parallel group of plates was fixed in 4% paraformaldehyde/PBS and DAPI stained, and image-based detection of intracellular parasites was collected with the In Cell 2000 High Content Analyzer (GE Healthcare). For the siRNA pools confirmed in the multiplexed plate readout as having a significant impact on intracellular *T. cruzi* growth, images were used to obtain data for the relative number of intracellular parasites/infected cell with manual confirmation. These distributions were compared to the nontargeting siRNA control group with the Mann-Whitney test. In some instances, binned distributions were plotted for clarity.

Pyruvate Dehydrogenase Activity

HeLa cells were reverse transfected with 25 nM siRNA in a 6-well plate. At 24 hr posttransfection, media was exchanged for D2 and at 48 hr was exchanged again for D2 (without pyruvate and phenol indicator). At 72 hr posttransfection, cells were washed with PBS, and lysates were collected in 80 μl provided sample buffer containing Complete Mini-EDTA free protease inhibitor cocktail (Roche) on ice. Protein concentration in clarified lysates (3,000 rpm spin for 10 min) was measured with the DC Protein Assay (BioRad) and adjusted to the same concentration in 50 μl. Pyruvate dehydrogenase activity was measured in each sample with a PDH activity assay kit (Abcam) according to the manufacturer's instructions. Assays were read on an Epson photo quality scanner, and densitometry for each band was calculated with Alpha View software version 3.1.1.0 (Alpha Innotech).

β Oxidation Assay

Transfected cells (as above) were loaded with 2 μCi ^{3}H-palmitate (Perkin Elmer) for 2 hr in media containing 2% fatty acid-free BSA, 0.25 mM carnitine, and low glucose DMEM. Resulting ^3H_2O released from β oxidation into the

(G) Intracellular CFSE-labeled *T. cruzi* amastigotes measured by flow cytometry reveal a uniform undivided population at 18 hpi (generation = 0, red line) with overlapping intensity to extracellular CFSE-labeled parasites fixed at time 0 (blue) (left panel) and five distinct generations at 48 hpi in untreated HFFs (middle panel). AktVIIIi (1 μM) added at 18 hpi inhibits *T. cruzi* proliferation (right panel).

(H) Acute silencing of *PRKAB1*, the regulatory subunit of AMPK, enhances intracellular growth of *T. cruzi* as confirmed in secondary screens.

(I) Constitutive mTORC1 activation in stable *TSC2* knockdown HeLa cells (shTSC2) enhances *T. cruzi* growth (measured at 48 hpi) as compared to control cells expressing shRNA to firefly luciferase (shLUC) and is sensitive to rapamycin (500 nM) and AktVIIIi (2 μM) added at 18 hpi.

(F and I) Lines indicate the parasite/cell median. Significance was determined by Kruskal-Wallis and Dunn's Mulitple Comparison tests (***$p < 0.001$). Experiments shown are representative of two to three independent observations.

(J) Proposed interaction of *T. cruzi* with Akt-activated mTORC1-dependent and -independent signaling pathways. siRNA-mediated depletion of host gene resulting in decreased intracellular *T. cruzi* growth (yellow) or increased growth (blue) is shown.

See also Figure S3.

supernatant was cleared of residual labeled palmitate/BSA through sequential extractions with 10% TCA, 5% TCA/10% BSA, and chloroform:methanol. Supernatant was collected, added to 5 ml Ecolume scintillation fluid (MP Biomedicals), and radioactivity was measured. All activity measurements were normalized to total protein quantified from cell lysates.

Biochemical Supplementation

HeLa cells (20,000) were reverse transfected, as indicated above, with 25 nM control or *TPRT*- or *GCH1*-targeting siRNA (Dharmacon/Thermo Fisher Scientific, Lafayette, CO) and seeded onto 10 mm round coverslips (Electron Microscopy Sciences, Hatfield, PA). At 18 hr posttransfection, media was exchanged for D2 supplemented with CoQ_{10} (Sigma-Aldrich, St. Louis, MO) (solubilized in ethanol to 5 mM, diluted in FCS, then in serum-free DMEM to final concentrations of 5 μM CoQ_{10} and 2% FCS), 10 μM Uridine (Sigma-Aldrich), 1 mM N-acetylcysteine (NAC) (Sigma-Aldrich), 7,8 dihydro-L-biopterin (Caymen Chemicals, Ann Arbor, MI), or vehicle controls. At 48 hr posttransfection, cells were infected with *T. cruzi* (MOI = 5) for 2 hr, washed twice to remove unattached parasites, and left in supplemented media. At 72 hr postinfection, cells were rinsed with sterile PBS and fixed in 2% paraformaldehyde/PBS. Parasite and host nuclei were DAPI stained, and parasites per infected cell were counted by fluorescent microscopy with an Eclipse TE300 (Nikon Instruments Inc., Melville, NY) under 60× magnification.

T. cruzi Growth Inhibition

Parasite-infected cells were treated at 18 hpi with AktVIIIi (EMD Chemicals, Cambridge, MA) or rapamycin (Tocris, Minneapolis, MN), and relative infection was determined in a multiplexed plate assay as described above. Intracellular *T. cruzi*/infected cell was determined for a minimum of 100 cells after visualization/counting of parasite and host cell nuclei with DAPI. The median number of intracellular parasites was compared to the corresponding untreated control with a Kruskal-Wallis test followed by Dunn's Multiple Comparison test.

T. cruzi Amastigote Proliferation Assay

Freshly isolated *T. cruzi* trypomastigotes were labeled with 1.5 μM CFSE from the CellTrace CFSE Cell Proliferation Kit (Life Technologies) per the manufacturer's protocol. Subconfluent monolayers of HFFs were infected with CFSE-labeled parasites to achieve one parasite/infected cell for most infected cells prior to the first parasite replication event. Monolayers were washed extensively and cells harvested by scraping and five passages through a 26 gauge needle to liberate the intracellular amastigotes at both 18 hpi (prereplication) and 48 hpi (postreplication). Cellular debris was removed by centrifugation. Relative CFSE fluorescence was measured for ~10,000 parasites by LSRFortessa (BD Biosciences), and proliferation was plotted and analyzed by FloJo Data Analysis Software (Tree Star, Inc.).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.11.011.

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